Exonuclease activity is required for sequence addition and Cdc13p loading at a de novo telomere

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The *Saccharomyces cerevisiae* Mre11p/Rad50p/Xrs2p (MRX) complex is evolutionarily conserved and functions in DNA repair and at telomeres [1–3]. In vivo, MRX is required for a 5′ → 3′ exonuclease activity that mediates DNA recombination at double-strand breaks (DSBs). Paradoxically, abolition of this exonuclease activity in MRX mutants results in shortened telomeric DNA tracts. To further explore the role of MRX at telomeres, we analyzed MRX mutants in a de novo telomere addition assay in yeast cells [4]. We found that the MRX genes were absolutely required for telomerase-mediated addition in this assay. Furthermore, we found that Cdc13p, a single-stranded telomeric DNA binding protein essential for telomere DNA synthesis and protection [5], was unable to bind to the de novo telomeric DNA substrate in cells lacking Rad50p. Based on the results from this model system, we propose that the MRX complex helps to prepare telomeric DNA for the loading of Cdc13p, which then protects the chromosome from further degradation and recruits telomerase and other DNA replication components to synthesize telomeric DNA.

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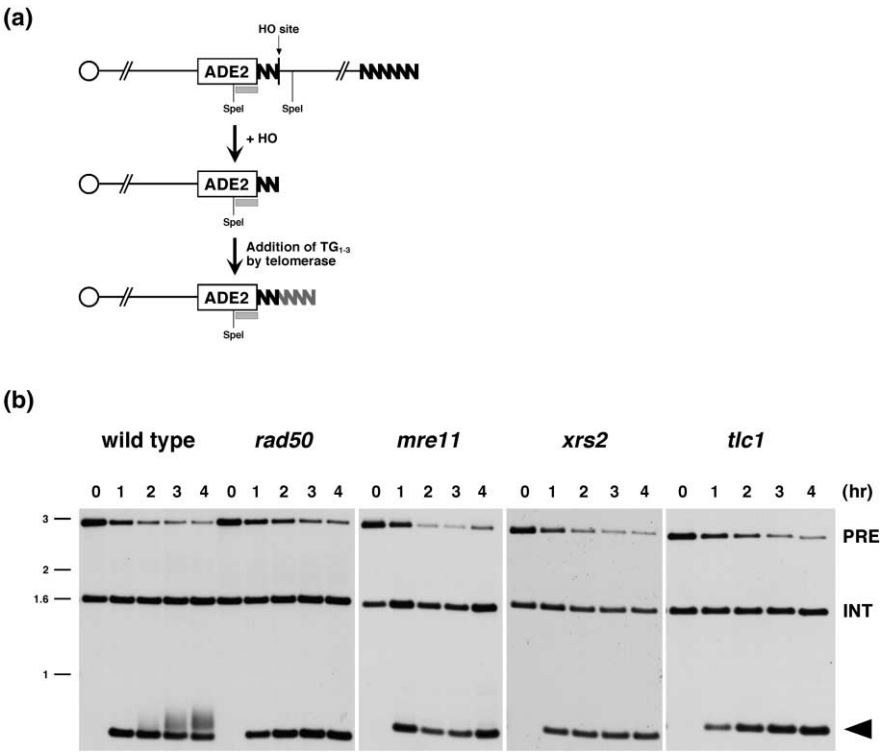
Results and discussion

Telomeres are essential for the stability of linear eukaryotic genomes—they protect chromosome ends from degradation by nucleases, prevent end-to-end fusions, and are required for the complete replication of chromosomes [6]. In *Saccharomyces cerevisiae* there are about 30 known genes that affect the length of telomere DNA tracts, both positively and negatively [7, 8]. The role some of these genes play in telomere biology is well understood. Telomerase, for example, synthesizes telomeric DNA and is composed of a protein catalytic subunit with homology to reverse transcriptases (Est2p) and an RNA component (Tlc1) that serves as a template for telomeric DNA production. Cdc13p binds to single-stranded, G-rich telomeric DNA in vitro [9, 10] and is required to protect the end of the chromosome from degradation [11] and to recruit telomerase activity and DNA polymerases for telomeric DNA synthesis [12, 13]. However, less is known about the mechanism by which many of the gene products involved in telomere maintenance function. One group of genes functions not only at telomeres but also at double-strand breaks (DSBs) in the cell. Mutations in *RAD50*, *MRE11*, or *XRS2* result in mitotic defects in the repair of DSBs by homologous recombination or nonhomologous end joining, as well as telomeres that are very short [1]. The yeast Mre11p/Rad50p/Xrs2p (MRX) complex and its human homolog have been shown to possess single-stranded endonuclease, single-stranded and double-stranded 3′ → 5′ exonuclease, and DNA unwinding activities in vitro [14]. The MRX complex in vivo, however, is required for the 5′ → 3′ resection of DSBs in yeast [1]. To explain this apparent contradiction, some researchers have suggested that first the MRX complex unwinds DNA, and then the endonuclease functions to produce a 3′ single-stranded tail [14]. Based on epistasis analysis, the MRX complex is in the same pathway for maintaining telomeric DNA as telomerase [15]. Since telomerase does not use double-stranded blunt-end DNA as a substrate in vitro [16], it has been proposed that the MRX complex prepares a 3′ single-stranded tail at the end of a telomere for telomerase to use as a substrate [15].

To better understand the role of the MRX complex in telomere maintenance, we used an assay that sensitively monitors telomerase-mediated telomere addition at a de novo telomere in *S. cerevisiae* cells [4] (Figure 1a). The assay is initiated by induction of the HO endonuclease, which cleaves a chromosome immediately adjacent to an internal tract of telomeric DNA sequence. This new end rapidly becomes a fully functional telomere; it is protected from degradation in a *CDC13*-dependent manner, and telomere DNA sequences are added to the end, dependent upon all the known telomerase-associated gene products [4]. When wild-type cells are arrested and held in M phase by using nocodazole, telomerase- (i.e., *TLC1*) dependent sequence addition is detected at the de novo telomere ([4] and Figure 1b). Unexpectedly, when strains lacking *RAD50*, *MRE11*, or *XRS2* were assayed, telomere addition was not detectable (Figure 1b). This result suggested that the activity of the MRX complex was needed

Figure 1



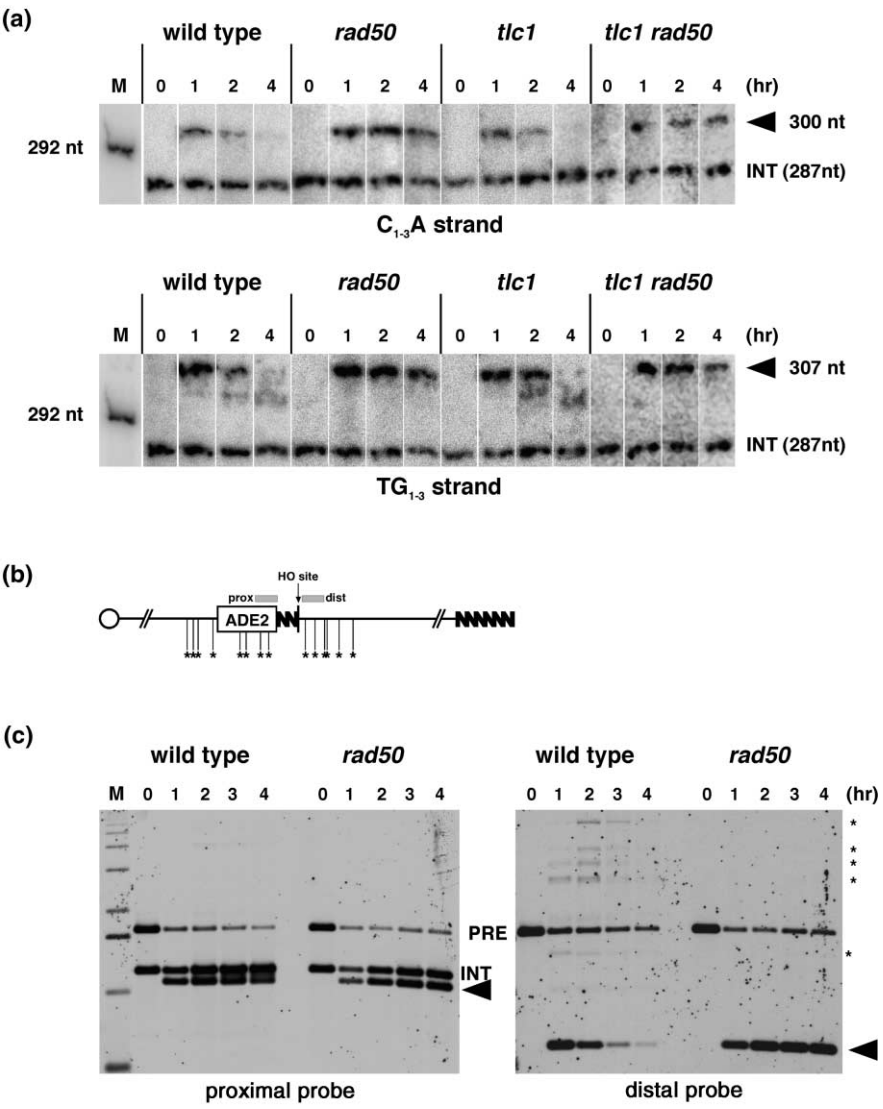
MRE11/RAD50/XRS2 are required for an early step in telomerase-mediated sequence addition. **(a)** Schematic representation of the de novo telomere addition assay (not to scale). The *ADH4* locus was replaced in a haploid yeast strain with a 6 kbp fragment consisting of the *ADE2* gene, 81 bp of TG_{1-3} sequence (zigzag line), and the recognition site for the HO endonuclease, as previously described [4]. HO is induced by the addition of galactose to the media. The *ADE2* probe used to monitor the TG_{1-3}/HO end is shown as a gray box. **(b)** Wild-type (UCC5913), *rad50* (UCC8000), *mre11* (UCC5969), *xrs2* (UCC5992), and *tlc1* (UCC5961) cells were analyzed with the de novo telomere addition assay [4]. Cells were arrested and held in M phase by using nocodazole. HO expression was induced by using galactose, and samples were taken every hour. A Southern blot analysis of SpeI-cut genomic DNA probed with part of the *ADE2* gene is shown. The band-labeled PRE represents the 3 kbp SpeI fragment from the construct on chromosome VII-L. After cleavage with HO, this fragment is converted into a new band (~0.7 kbp, marked with the arrowhead). The *ADE2* probe also detects a 1.6 kb SpeI fragment from the *ade2-101* locus. This band is marked INT and serves as a DNA loading control.

for telomerase to synthesize telomeric DNA onto the de novo telomere.

The MRX-dependent nuclease activity may be needed to prepare the de novo telomere DNA as a substrate for telomerase [15]. To test this idea, we subjected cells with and without *RAD50* to the telomere addition assay and examined the de novo telomere at nucleotide resolution on a denaturing polyacrylamide gel. When the $C_{1-3}A$ strand (strand with 5′ end) was analyzed in wild-type cells, a band corresponding to the newly created end is only faintly detected after 4 hr (Figure 2a). The lack of signal is partly due to the addition of telomeric sequences that are not resolved on this gel. When *tlc1* cells (where no telomere addition can occur) were examined, the band was undetectable at 4 hr, indicating that the $C_{1-3}A$ strand was degraded (Figure 2a). In stark contrast, the 5′ end was very stable at 4 hr in *rad50* or *rad50 tlc1* cells (Figure 2a). When the TG_{1-3} strand (3′ end) was analyzed, wild-type and *tlc1* cells had limited degradation of the end since the signal was still detectable as a smear that extended ~10 nucleotides below the initial 307 nucleotide length of the de novo telomere (Figure 2a). In the *rad50* and *rad50 tlc1* cells, the TG_{1-3} strand, once again, was quite stable (Figure 2a). Thus, it appears that 5′ → 3′ processing is a prerequisite for 3′ → 5′ processing (reflected in the TG_{1-3} stability observed in *rad50* cells). Taking all these data together, we conclude that Rad50p greatly facilitates degradation of the 5′ end strand at the de novo telomere, as has been shown at DSBs [1]. That is, Rad50p promotes formation of a 3′ TG_{1-3} single-stranded tail.

An earlier study that examined exonuclease activity at an HO-induced DSB in actively dividing cells reported that, compared to wild-type, a *rad50* mutation slowed the rate of degradation by only 2-fold [17]. This contrasts with what we observed at the de novo telomere, where virtually no degradation of the end is detected in *rad50* cells (Figure 2a). To determine if the lack of degradation of a *rad50* strain in our assay was unique to the de novo telomere end, we examined the other side (nontelomeric DNA) of the DSB. The telomere addition assay was performed on wild-type and *rad50* cells, and DNA from the TG_{1-3} side (proximal in Figure 2b) and the nontelomeric side (distal in Figure 2b) of the DSB was analyzed. As previously shown in wild-type cells [4], DNA from the nontelomeric side of the DSB was rapidly degraded, as shown by both the appearance of slowly migrating bands over time (a result of 5′ → 3′ single-strand degradation) and the loss of signal intensity of the initial band produced by HO cleavage (Figure 2c). In contrast, when the nontelomeric side of the DSB from *rad50* cells was examined, no DNA degradation whatsoever was detected over the time course of the experiment. We conclude that when cells are arrested in M phase, as in our experiments here, virtually all the exonuclease activity at a DNA end requires the

Figure 2



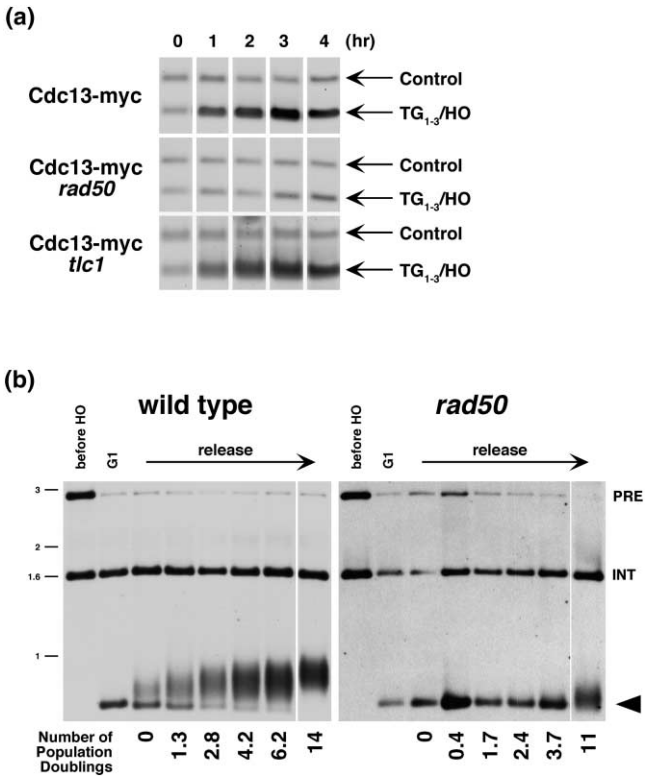
RAD50 is required for resection of DNA ends in the de novo telomere assay. **(a)** DNA samples described in Figure 1 plus DNA processed identically from *rad50 tlc1* (UCC8024) cells were analyzed on denaturing polyacrylamide gels (5%) and electroblotted. Southern-blot analysis of DdeI-cut genomic DNA probed with a single-stranded riboprobe that recognizes either the TG_{1-3} or $C_{1-3}A$ strand is shown. After cleavage by HO endonuclease, a 307 nt fragment (riboprobe that recognizes TG_{1-3} strand) or a 300 nt fragment (riboprobe that recognizes $C_{1-3}A$ strand) containing the TG_{1-3}/HO end is generated (band marked with the arrowhead). The riboprobes also detect a 287 nt DdeI fragment from the *ade2-101* locus. This band is marked INT and serves as a DNA loading control. **(b)** Schematic representation of SspI sites on chromosome VII. SspI sites are depicted (*), and the location of the proximal and distal probes used in (c) are shown as gray boxes (not drawn to scale). **(c)** DNA was isolated from nocodazole-arrested wild-type (UCC5913) and *rad50* (UCC8000) cells and digested with SspI. A Southern blot was first probed with a fragment that recognizes sequence distal to the HO site (distal probe). The band labeled PRE represents the 1.733 kbp SspI fragment from the construct on chromosome VII. After cleavage with HO, this fragment is converted into a new band (~1.1 kb as detected with the proximal probe and ~0.6 kb with the distal probe, marked with arrowheads). The proximal probe also detects a 1.2 kbp SspI fragment from the *ade2-101* locus (marked INT). Slowly migrating bands (*) are generated when 5′ → 3′ single-stranded degradation progresses through the recognition site for SspI, preventing the enzyme from cleaving the resulting single-stranded DNA [4]. The blot was then stripped and reprobed with the *ADE2* probe (proximal probe).

MRX complex, while in cycling cells there are additional pathways that facilitate resection [17].

One explanation for a lack of telomere addition in a *rad50* strain is that the 5′ → 3′ strand DNA degradation may be required for loading Cdc13p. To test whether Cdc13p bound the de novo telomere end in the absence of Rad50p, we performed chromatin immunoprecipitation (ChIP) on cells carrying an epitope-tagged version of Cdc13p (Cdc13p-Myc). As shown in Figure 3a, Cdc13p-Myc was clearly present at the de novo telomere in wild-type cells but was detectable at much lower levels after 4 hr in *rad50* cells. It was formally possible that the loading of Cdc13p onto the de novo telomere was facilitated by telomerase-mediated extension of the 3′ TG_{1-3} strand. However, this was not the case, because Cdc13p-Myc still bound to the de novo telomere in cells lacking *TLC1* (Figure 3a). We conclude that the 5′ → 3′ exonuclease activity promoted by the MRX complex presents a 3′ TG_{1-3} tail substrate for Cdc13p to bind at the de novo telomere.

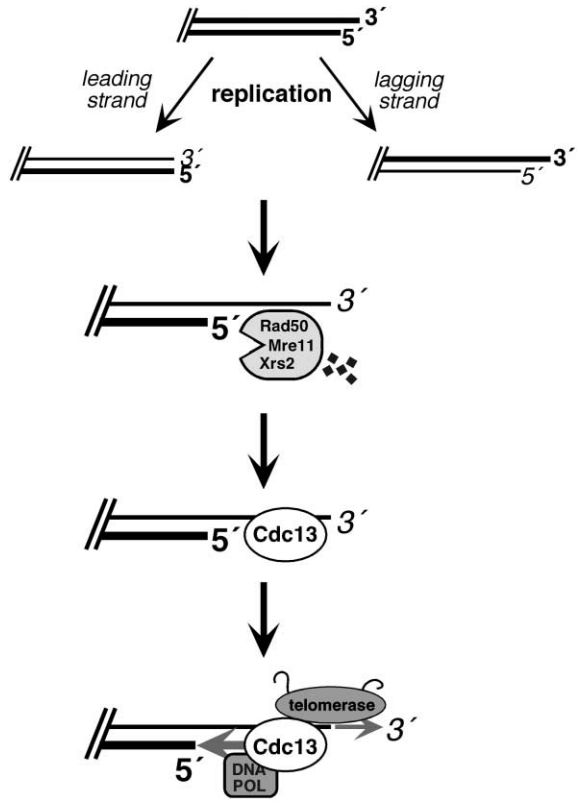
If the de novo telomere assay, as carried out above, represents all aspects of native telomere replication and formation, then it would be expected that *rad50* cells are incapable of maintaining telomeric DNA sequences and should undergo senescence, just as a *tlc1* strain does. In fact, this has been reported to occur in one strain background [18]. However, most *rad50* strains, including the ones used in this study, do not have a senescent phenotype. Therefore, another, less efficient pathway may be involved in facilitating telomere addition at native chromosome ends. Indeed, when the de novo telomere was created in G1-arrested cells, and the cells were then released into the

Figure 3



Rad50p-mediated degradation of the TG_{1-3}/HO end promotes loading of Cdc13p and is required for efficient telomere addition in dividing cells. **(a)** Wild-type (UCC8005), *rad50* (UCC8019), and *tlc1* (UCC8023) cells containing Cdc13p-Myc were treated as in Figure 1, crosslinked by using formaldehyde, and subjected to ChIP analysis. The DNA from the immunoprecipitates were PCR amplified by using primers specific for sequence adjacent to the TG_{1-3}/HO end (234 bp product) and primers located ~47 kbp internal to the natural telomere on VII-L (control, 312 bp product). Products were run on a 2% agarose gel, stained with Vistra Green (Amersham Pharmacia Biotech), and visualized with a Molecular Dynamics Typhoon Imager. **(b)** Wild-type (UCC5913) and *rad50* (UCC8000) cells were arrested and held in G1 by using the mating pheromone α factor, galactose was added to induce *HO* expression for 2 hr, and cells were resuspended in prewarmed YEPD to release. Samples were taken for DNA analysis and cell counting every 2 hr for 12 hr, and then cells were diluted to allow 7–8 more generations of growth. Strains lacking *RAD50* do not undergo as many cell divisions as wild-type since they arrest for a longer period of time in G2/M after HO cutting.

Figure 4



Model for MRX-mediated processing of telomeric DNA. The end of a chromosome after leading- and lagging-strand replication is shown. Leading-strand synthesis results in a blunt-end molecule, while lagging-strand synthesis leaves a 3′ single-strand overhang of undetermined length once the RNA primer is removed. Processing by the MRX complex is required for Cdc13p binding of the blunt-end and may also be needed for efficient loading of Cdc13p onto the end of the lagging-strand product. Once Cdc13p binds, it prevents further degradation by the MRX complex and recruits telomerase [12] and DNA lagging-strand replication machinery for telomeric DNA synthesis [4, 13].

cycle, sequence addition was evident in wild-type cells before they had all divided (Figure 3b). However, telomere addition was barely detected at the de novo end in *rad50* cells, even after four cell doublings (Figure 3b). Despite this long delay, a functional telomere was created, and telomere addition was eventually detected after overnight growth of the culture. This telomere addition was not simply the result of a recombination event because *rad50* cells lacking the *RAD52* gene (required for homologous recombination in yeast) also eventually added a telomere after overnight growth (data not shown). Thus, in dividing cells, Rad50p is not absolutely required for telomere addition; rather, it makes addition occur more effectively.

Presumably, a different, cell-cycle-regulated nuclease activity and/or progression of a replication fork through a telomere creates a 3′ TG_{1-3} tail for Cdc13p binding and telomere addition. We speculate that, compared to that carried out by the MRX complex, Cdc13p loading by this secondary mechanism may either be inefficient or not properly coordinated with telomerase addition activity. A similar inefficient phenomenon occurs in mating-type switching in *rad50* cells; exonucleolytic processing of the DSB is retarded in dividing cells, but switching (recombination) eventually occurs [19]. The results presented here, in a de novo telomere assay, support the idea that the

role of the MRX complex at telomeres is to prepare the end of the chromosome for the binding of Cdc13p. This may be particularly germane when the chromosome has been replicated to the end by conventional leading- and lagging-strand synthesis; one of the products will have a blunt end, while the other will have a short 3′ overhang generated by the removal of the RNA primer (~10 nt) [20] used in lagging-strand synthesis (Figure 4). The MRX complex creates a 3′ single-strand overhang on the blunt end and may also work on the lagging-strand product to promote efficient binding of Cdc13p. It is plausible that having multiple Cdc13p molecules bound at an end makes Cdc13p more effective in recruiting telomerase and protecting the telomere.

The human homolog of the *S. cerevisiae* MRX complex was found to associate with the telomeric binding protein TRF2 [3]. Mammalian telomeres have been shown to form "t loops," where the 3′ telomeric overhang invades duplex telomeric DNA to create a stable heteroduplex at the base of the loop [21]. TRF2 binds to this heteroduplex and is thought to stabilize it. In addition, a human protein has recently been identified that binds to the 3′ telomeric overhang, analogous to Cdc13p binding at yeast telomeres [22]. Similar to what we show for a yeast de novo telomere, the human equivalent of the MRX complex may help to process the end of the chromosome to produce the 3′ telomeric overhang needed for t loop formation and/or binding by the human Cdc13p-like protein.

Material and methods

Strains

UCC5913 (*MATa-inc ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1:GAL1-HO-LEU2 VII-L::ADE2-TG(1-3)-HO site-LYS2*) and UCC5961 (UCC5913 *tlc1::HIS3*) are segregants from the sporulation of UCC5695 and served as parents for all strains created in this study [4]. All gene disruptions precisely deleted the open reading frame (ORF) and were performed by PCR-mediated transformation [23]. UCC8005, UCC8019, and UCC8023 were made by epitope tagging Cdc13p on the C terminus via PCR-mediated transformation [24] of UCC5913, UCC8000, and UCC5961, respectively.

ChIP

Chromatin immunoprecipitation was performed as described [25]. The DNA from the immunoprecipitates were PCR amplified for 28 cycles by using primers XIP_1 5′-CTCTACTCTCGGGGATCCGTCTCGG-3′ and XIP_2 5′-ACCCTGCCCATTACCTCCCAGAATCC-3′ specific for sequence adjacent to the TG_{1-3}/HO end, and primers XIPi_1 5′-ACATATT GGACATGCCAAAGCTGCC-3′ and XIPi_2 5′-CGATCTCTTCTGG CAGAAGCAACACC-3′ located ~47 kbp internal to the natural telomere on VII-L.

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